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Characterization of a Novel Type of Serine/Threonine Kinase That Specifically Phosphorylates the Human Goodpasture Antigen*

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Goodpasture disease is an autoimmune disorder that occurs naturally only in humans. Also exclusive to humans is the phosphorylation process that targets the unique N-terminal region of the Goodpasture antigen. Here we report the molecular cloning of GPBP (Goodpasture antigen-binding protein), a previously unknown 624-residue polypeptide. Although the predicted sequence does not meet the conventional structural requirements for a protein kinase, its recombinant counterpart specifically binds to and phosphorylates the exclusive N-terminal region of the human Goodpasture antigen *in vitro*. This novel kinase is widely expressed in human tissues but shows preferential expression in the histological structures that are targets of common autoimmune responses. The work presented in this report highlights a novel gene to be explored in human autoimmunity.

Goodpasture (GP)[1] disease is an autoimmune disorder described only in humans. In GP patients autoantibodies against the non-collagenous C-terminal domain (NC1) of the α3 chain of collagen IV cause a rapidly progressive glomerulonephritis and often lung hemorrhage, the two cardinal clinical manifestations of the GP syndrome (see Ref. 1 for review). Since the NC1 domain is a highly conserved domain among species and between the different collagen IV α chains (α1–α6) (2), the exclusive involvement of the human α3(IV)NC1 in a natural autoimmune response suggests that this domain has structural and/or biological peculiarities of pathogenic relevance. Consistent with this, the N terminus of the human antigen is highly divergent, and it contains a unique 5-residue motif, KRGDS[9], that conforms to a functional phosphorylation site for type A protein kinases (3, 4). Furthermore, the corresponding human gene, but not the other human related or homologous genes from other species, generates multiple transcripts by an exclusive alternative splicing phenomenon (5–7). Recent studies indicate that the phosphorylation of the N terminus of the GP antigen by cAMP-dependent protein kinase is up-regulated by the presence of the alternative products.[2] Thus, specific serine/threonine phosphorylation appears to be a major biological difference between the human antigen, antigen from other species, and the homologous domains from other human α(IV) chains and therefore might be important in pathogenesis (1, 4).

Here we report the cloning and characterization of a novel type of serine/threonine kinase that specifically binds to and phosphorylates the unique N-terminal region of the human GP antigen.

MATERIALS AND METHODS

Synthetic Polymers

Peptides—GPpep1, KGKRGDSGSPATWTTRGFVFT, representing residues 3–23 of the human GP antigen, and GPpep1Ala[9], KGKRGDAGSPATWTTRGFVFT, a mutant Ser[9] to Ala[9], were synthesized by MedProbe and CHIRON. FLAG peptide, DYKDDDDK, was from Sigma.

Oligonucleotides—The following as well as several other GPBP-specific oligonucleotides were synthesized by Genosys and Life Technologies, Inc:. ON-GPBP-54m, TCGAATTCACCATGGCCCCACTAGCCGACTACAAGGACGACGATGACAAG and ON-GPBP-55c, CCGAGCCCGACGAGTTCCAGCTCTGATTATCCGACATCTTGTCATCGTCG; ON-HNC-B-N-14m, CGGGATCCGCTAGCTAAGCCAGGCAAGGATGG; ON-HNC-B-N-16c, CGGGATCCATGCATAAATAGCAGTTCTGCTGT.

Isolation and Characterization of cDNA Clones Encoding Human GPBP

Several human λ-gt11 cDNA expression libraries (eye, fetal and adult lung, kidney, and HeLa S3, from CLONTECH) were probed for cDNAs encoding proteins interacting with GPpep1. Nitrocellulose filters (Millipore) prepared following standard immunoscreening procedures were blocked and incubated with 1–10 nmol per ml of GPpep1 at 37 °C. Specifically bound GPpep1 was detected using M3/1A monoclonal antibodies (7). A single clone was identified in the HeLa-derived library (HeLa1). Specificity of fusion protein binding was confirmed by similarly binding recombinant eukaryotic human GP antigen. The *Eco*RI cDNA insert of HeLa1 (0.5 kb) was used to screen further the same library and to isolate overlapping cDNAs. The largest cDNA (2.4 kb) containing the entire cDNA of HeLa1 (n4′) was fully sequenced.

Northern and Southern Blots

Pre-made Northern and Southern blots (CLONTECH) were probed with HeLa1 cDNA following the manufacturers' instructions.

Plasmid Construction, Expression, and Purification of Recombinant Proteins

GPBP-derived Material—The original λ-gt11 HeLa1 clone was expressed as a lysogen in *Escherichia coli* Y1089 (8). The corresponding β-galactosidase-derived fusion protein containing the N-terminal 150 residues of GPBP was purified from the cell lysate using an APTG-agarose column (Roche Molecular Biochemicals). The *Eco*RI 2.4-kb fragment of n4′ was subcloned in Bluescribe M13+ vector (Stratagene)

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF136450.

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[1] The abbreviations used are: GP, Goodpasture; bp, base pair; GPBP and rGPBP, native and recombinant Goodpasture antigen-binding protein; GST, glutathione *S*-transferase; HLA, human lymphocyte antigens; kb, kilobase pairs; NC1, non-collagenous domain; PH, pleckstrin homology; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; ORF, open reading frame.

[2] J. Saus, manuscript in preparation.

This paper is available on line at http://www.jbc.org

(BS-n4'), amplified, and used for subsequent cloning. A DNA fragment containing from 5' to 3', an *Eco*RI restriction site, a standard Kozak consensus for translation initiation, a region coding for a tag peptide sequence (FLAG, DYKDDDDK), and the sequence coding for the first 11 residues of GPBP including the predicted Met, and a *Ban*II restriction site, was obtained by hybridizing ON-GPBP-54m and ON-GPBP-55c and extending with modified T_7 DNA polymerase (Amersham Pharmacia Biotech). The resulting DNA product was digested with *Eco*RI and *Ban*II and ligated with the *Ban*II/*Eco*RI cDNA fragment of BS-n4' in the *Eco*RI site of pHIL-D2 (Invitrogen) to produce pHIL-FLAG-n4'. This plasmid was used to obtain Mut$^+$ transformants of the GS115 strain of *Pichia pastoris* and to express FLAG-tagged recombinant GPBP (rGPBP) either by conventional liquid culture or by fermentation procedures (*Pichia* Expression Kit, Invitrogen). The cell lysates were loaded onto an anti-FLAG M2 column (Sigma), the unbound material washed out with Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) or salt-supplemented TBS (up to 2 M NaCl), and the recombinant material eluted with FLAG peptide. For expression in cultured human kidney-derived 293 cells (ATCC 1573-CRL), the 2.4- or 2.0-kb *Eco*RI cDNA insert of either BS-n4' or pHIL-FLAG-n4' was subcloned in pcDNA3 (Invitrogen) to produce pc-n4' and pc-FLAG-n4', respectively. When used for transient expression, 18 h after transfection the cells were lysed with 3.5–4 μl/cm^2 chilled lysis buffer (1% Nonidet P-40 or Triton X-100, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride in TBS) with or without 0.1% SDS depending whether the lysate was to be used for SDS-PAGE or FLAG purification, respectively. For FLAG purification, the lysate of four to six 175-cm^2 culture dishes was diluted up to 50 ml with lysis buffer and purified as above. For stable expression, the cells were similarly transfected with pc-n4' and selected for 3 weeks with 800 μg/ml G418. For bacterial recombinant expression, the 2.0 kb *Eco*RI cDNA fragment of pHIL-FLAG-n4' was cloned in-frame downstream of the glutathione *S*-transferase (GST)-encoding cDNA of pGEX 5x 1 (Amersham Pharmacia Biotech). The resulting construct was used to express GST-GPBP fusion protein in DH5α (9).

GP Antigen derived Material—Human recombinant GP antigen (rGP) was produced in 293 cells using the pRc/CMV-BM40 expression vector containing the α3-specific cDNA between ON-HNC-B-N-14m and ON-HNC-B-N-16c. The expression vector is a pRc/CMV (Invitrogen)-derived vector provided by Billy G. Hudson that contains cDNA encoding an initiation Met, a BM40 signal peptide followed by a tag peptide sequence (FLAG), and a polylinker cloning site. To obtain α3-specific cDNA, a polymerase chain reaction was performed using the oligonucleotides above and a plasmid containing the previously reported α3(IV) cDNA sequence (3) as template (clone C2). For stable expression of rGP, 293 cells were transfected with the resulting construct (fα3VLC) and selected with 400 μg/ml G418. The harvested rGP was purified using an anti-FLAG M2 column.

All the constructs were verified by restriction mapping and nucleotide sequencing.

Cell Culture and DNA Transfection

Human 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed using the calcium phosphate precipitation method of the Profection Mammalian Transfection Systems (Promega). Stably transfected cells were selected by their resistance to G418. Foci of surviving cells were isolated, cloned, and amplified.

Antibody Production

Polyclonal Antibodies Against the N-terminal Region of GPBP—Cells expressing HeLa1 λ-gt11 as a lysogen were lysed by sonication in the presence of Laemmli sample buffer and subjected to electrophoresis in a 7.5% acrylamide preparative gel. The gel was stained with Coomassie Blue, and the band containing the fusion protein of interest was excised and used for rabbit immunization (10). The antiserum was tested for reactivity using APTG affinity purified antigen. To obtain affinity purified antibodies, the antiserum was diluted 1:5 with TBS and loaded onto a Sepharose 4B column containing covalently bound affinity purified antigen. The bound material was eluted and, unless otherwise indicated, used in the immunochemical studies.

Monoclonal Antibodies against GPBP—Monoclonal antibodies were produced essentially as previously reported (7) using GST-GPBP. The supernatants of individual clones were analyzed for antibodies against rGPBP.

In Vitro Phosphorylation Assays

About 200 ng of rGPBP were incubated overnight at 30 °C in 25 mM β-glycerol phosphate (pH 7.0), 0.5 mM EDTA, 0.5 mM EGTA, 8 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol, and 0.132 μM [γ-^{32}P]ATP, in the presence or absence of 0.5–1 μg of protein substrates or 10 nmol of synthetic peptides, in a total volume of 50 μl.

In Vivo Phosphorylation Assays

Individual wells of a 24-well dish were seeded with normal or with stably pc-n4'-transfected 293 cells. When the cells were grown to the desired density, a number of wells of the normal 293 cells were transfected with pc-FLAG-n4'. After 12 h the culture medium was removed, 20 μCi/well of $H_3{}^{32}PO_4$ in 100 μl of phosphate-free Dulbecco's modified Eagle's medium added, and incubation continued for 4 h. The cells were lysed with 300 μl/well of TBS containing 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 0.2 mM vanadate and extracted with specific antibodies and protein A-Sepharose. When anti-GPBP serum was used, the lysate was precleared using preimmune serum and protein A-Sepharose.

In Vitro Dephosphorylation of rGPBP

About 1 μg of rGPBP was dephosphorylated in 100 μl of 10 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, and 50 mM potassium acetate with 0.85 units of calf intestine alkaline phosphatase (Amersham Pharmacia Biotech) for 30 min at 30 °C.

Renaturation Assays

In-blot renaturation assays were performed using 1–5 μg of rGPBP as described previously (11).

Nucleotide Sequence Analysis

The cDNA sequences were performed by the dideoxy chain termination method using α-^{35}S-dATP, modified T_7 DNA polymerase (Amersham Pharmacia Biotech), and universal or GPBP-specific primers (8–10).

^{32}P-Phosphoamino Acid Analysis

Immunopurified rGPBP or high performance liquid chromatography gel filtration fractions therefrom containing the material of interest were phosphorylated, hydrolyzed, and analyzed in one- (4) or two-dimensional thin layer chromatography (12). When performing two-dimensional analysis, the buffer for the first dimension was formic acid:acetic acid:water (1:3.1:35.9) (pH 1.9), and the buffer for the second dimension was acetic acid:pyridine:water (2:0.2:37.8) (pH 3.5). Amino acids were revealed with ninhydrin and ^{32}P-phosphoamino acids by autoradiography.

Physical Methods and Immunochemical Techniques

SDS-PAGE and Western blotting were performed as in Ref. 4. Immunohistochemistry studies were done on human multi-tissue control slides (Biomeda, Biogenex) using the ABC peroxidase method (13).

Computer Analysis

Homology searches were carried out against the GenBank™ and SwissProt data bases with the BLAST 2.0 (14) at the NCBI server and against the TIGR Human Gene Index data base for expressed sequence tags using the Institute for Genomic Research server. The search for functional patterns and profiles was performed against the PROSITE data base using the ProfileScan program at the Swiss Institute of Bioinformatics (15). Prediction of coiled-coil structures was done at the Swiss Institute for Experimental Cancer Research using the program Coils (16) with both 21- and 28-residue windows.

RESULTS

Molecular Cloning of GPBP—To search for proteins specifically interacting with the divergent N-terminal region of the human GP antigen, a 21-residue peptide (GPpep1), encompassing this and flanking sequence, and specific monoclonal antibodies against it were combined to screen several human cDNA expression libraries. The screening of more than 5×10^6 phages was required to identify a single HeLa-derived recombinant encoding a fusion protein specifically interacting with GPpep1 without disturbing antibody binding.

By using the cDNA insert of the original clone (HeLa1), we isolated a 2.4-kb cDNA (n4') that contains 408 bp of 5'-untranslated sequence, an open reading frame (ORF) of 1872-bp encoding 624 residues, and 109-bp of 3'-untranslated sequence (Fig. 1). Other structural features are of interest. First, the

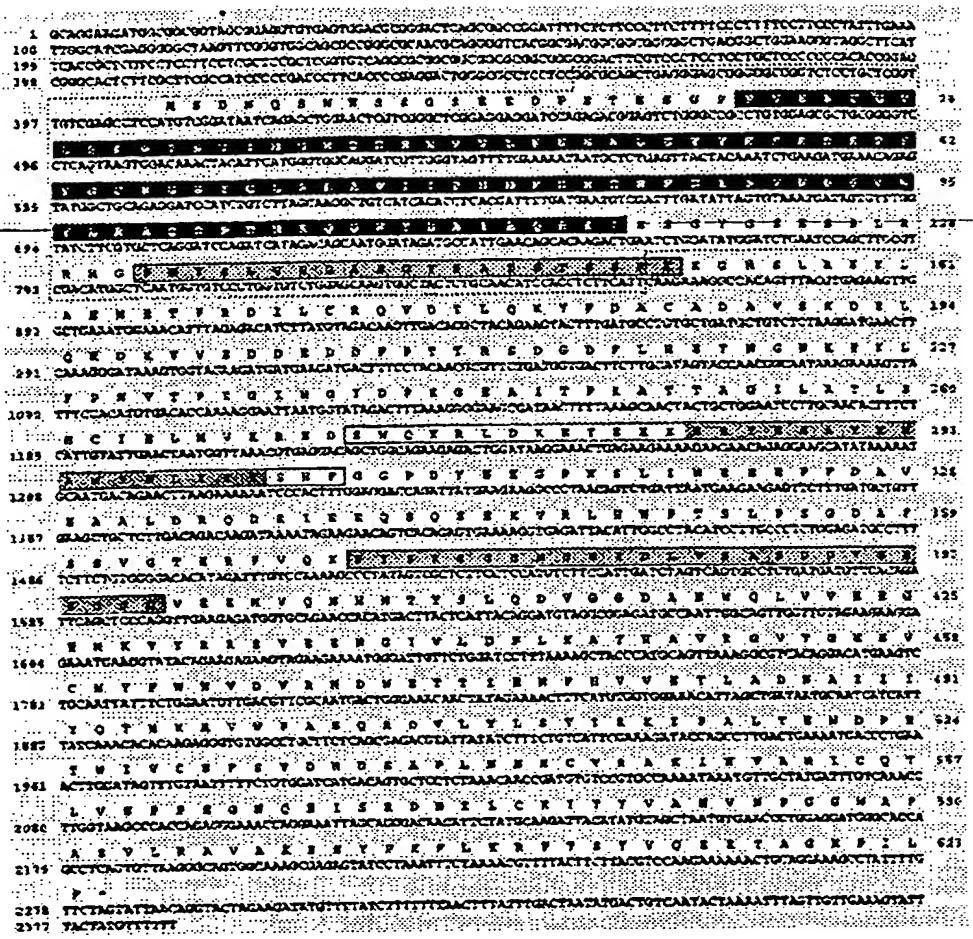

FIG. 1. **Nucleotide and derived amino acid sequences of n4'.** The denoted structural features are from 5'- to 3'-end and are as follows: the cDNA present in the original clone (HeLa1) (*dotted box*) that contains the PH homology domain (in *black*) and the Ser-Xaa-Yaa repeat (in *gray*); the heptad repeat of the predictable coiled-coil structure (*open box*) containing the bipartite nuclear localization signal (in *gray*); and a serine-rich domain (*filled gray box*). The *asterisks* denote the positions of in-frame stop codons.

predicted polypeptide has a large number of phosphorylatable (17.9%) and acidic (16%) residues with an unequal distribution along the sequence. Serine, which is the most abundant residue (9.3%), shows preference for two short regions of the protein where it comprises nearly 40% of the amino acids compared with an average of less than 7% throughout the rest of the polypeptide chain. It is also noteworthy that the more N-terminal serine-rich region consists mainly of a Ser-Xaa-Yaa repeat. Acidic residues are preferentially located at the three N-terminal quarters of the polypeptide with nearly 18% of the residues being acidic. These residues represent only 9% in the most C-terminal quarter of the polypeptide, resulting in a polypeptide chain with two electrically opposite areas. At the N terminus, the polypeptide contains a pleckstrin homology (PH) domain that has been implicated in the recruitment of many signaling proteins to the cell membrane where they exert their biological activities (17). Finally, a bipartite nuclear targeting sequence (18) exists as an integral part of a heptad repeat region that meets all the structural requirements to form a coiled-coil (16).

Protein data bank searches revealed homologies almost exclusively within the approximately 100 residues at the N-terminal region harboring the PH domain. The PH domain of the oxysterol-binding protein, which displays an overall identity of 33.5% and a similarity of 65.2% with our cloned protein, is the most similar. In addition, the *Caenorhabditis elegans* cosmid F25H2 (GenBank™ accession number Q93569) contains a hypothetical ORF that displays an overall identity of 26.5% and a similarity of 61% throughout the entire protein sequence indicating that similar proteins are present in lower invertebrates. Several tagged human expressed sequences (GenBank™ accession numbers AA287878, AA287561, AA307431, AA331618, AA040134, AA158618, AA040087, AA122226, AA158617, AA121104, AA412432, AA412433, AA282679, and N27578) demonstrated a high degree of nucleotide identity (above 98%) with the corresponding stretches of the GPBP cDNA, suggesting that they represent human GPBP. Interestingly, the tagged sequence AA287878 shows a gap of 67 nucleotides within the sequence corresponding to the GPBP 5'-untranslated region, suggesting that the GPBP pre-mRNA is alternatively spliced in human tissues (not shown).

The distribution and expression of the GPBP gene in human tissues was first assessed by Northern blot analysis (Fig. 2*A*). The gene is expressed as two major mRNAs species between 4.4- and 7.5-kb in length and other minor species of shorter lengths. The structural relationship between these multiple mRNA species is not known, and their relative expression varies between tissues. Striated muscle (skeletal and heart) is the tissue with highest expression, whereas lung and liver show the least.

Southern blot studies using genomic DNA from different species indicate that homologous genes exist throughout phylogeny (Fig. 2*B*). Consistent with the human origin of the probe, the hybridization intensities decrease in a progressive fashion as the origin of the genomic DNA moves away from humans in evolution.

Experimental Determination of the Translation Start Site—To confirm experimentally the predicted ORF, eukaryotic expression vectors containing either the 2.4-kb of cDNA of n4' or only the predicted ORF tagged with a FLAG sequence (Fig.

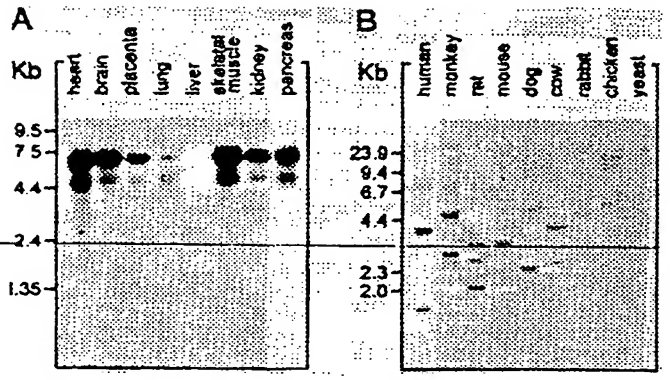

FIG. 2. **Distribution of GPBP in human tissues (Northern blot) and in eukaryotic species (Southern blot).** A random priming ^{32}P-labeled HeLa1 cDNA probe was used to identify homologous messages in a Northern blot of poly(A$^+$) RNA from the indicated human tissues (*A*) or in a Southern blot of genomic DNA from the indicated eukaryotic species (*B*). Northern hybridization was performed under highly stringent conditions to detect perfect matching messages and at low stringency in the Southern blot to allow the detection of messages with mismatches. No appreciable differences in the quality and amount of each individual poly(A$^+$) RNA were observed by denaturing gel electrophoresis and when probing with human β-actin cDNA a representative blot from the same lot. The *numbers* denote the position and the sizes in kb of the RNA or DNA markers used.

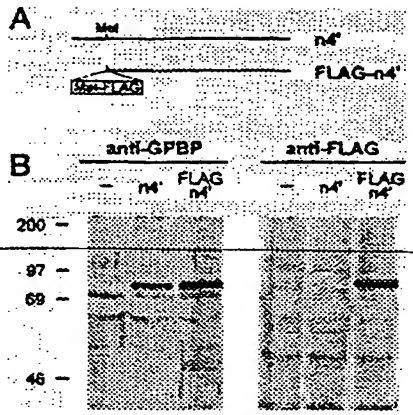

FIG. 3. **Experimental determination of the translation start site.** *A*, the two cDNAs present in pc-n4' and pc-FLAG-n4' plasmids used for transient expression are represented as *black lines*. The relative position of the corresponding predicted (n4') or engineered (FLAG-n4') translation start site is indicated (*Met*). *B*, the extracts from control (–), pc-n4'(n4'), or pc-FLAG-n4' (FLAG-n4') transfected 293 cells were subjected to SDS-PAGE under reducing conditions in 10% gels, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and blotted with the indicated antibodies. The *numbers* and *bars* indicate the molecular mass in kDa and the relative positions of the molecular weight markers, respectively.

3*A*) were used for transient expression assays in 293 cells. The corresponding extracts were analyzed by immunoblot using GPBP- or FLAG-specific antibodies. The GPBP-specific antibodies bind to a similar major polypeptide in both transfected cells, but only the polypeptide produced by the engineered construct expressed the FLAG sequence (Fig. 3*B*). This locates the translation start site of the n4' cDNA at the predicted Met and confirms the proposed primary structure. Furthermore, the recombinant polypeptides identified display a molecular mass higher than expected (80 *versus* 71 kDa) suggesting that GPBP undergoes post-translational modifications.

Expression and Characterization of Yeast rGPBP—Yeast expression and FLAG-based affinity purification were combined to produce rGPBP (Fig. 4*A*). A major polypeptide of ~89 kDa along with multiple related products displaying lower M_r were obtained. The recombinant material was recognized by both anti-FLAG and specific antibodies guaranteeing the fidelity of the expression system. Again, however, the M_r displayed by the major product was notably higher than predicted and even higher than the M_r of the 293 cell-derived recombinant material, supporting the idea that GPBP undergoes important and differential post-translational modifications. Since phosphorylatable residues are the most abundant in the polypeptide chain, we investigated the existence of phosphoamino acids in the recombinant materials. By using monoclonal or polyclonal (not shown) antibodies against phosphoserine (Ser(P)), phosphothreonine (Thr(P)), and phosphotyrosine (Tyr(P)), we identified the presence of all three phosphoresidues either in yeast rGPBP (Fig. 4*B*) or in 293 cell-derived material (not shown). The specificity of the antibodies was further assessed by partially inhibiting their binding by the addition of 5–10 mM of the corresponding phosphoamino acid (not shown). This suggests that the phosphoresidue content varies upon the cell expression system, and the M_r differences are mainly due to phosphorylation. Consistently, dephosphorylated yeast-derived material displays similar M_r to the material derived from 293 cells, and phosphoamino acid content correlates with SDS-PAGE mobilities (Fig. 4*C*). As an *in vivo* measurement, the phosphorylation of rGPBP in the 293 cells was assessed (Fig. 4*D*). Control cells (*lanes 1*) and cells expressing rGPBP in stable (*lanes 2*) or transient (*lanes 3*) modes were cultured in the presence of $H_3{}^{32}PO_4$. The recombinant material specifically immunoprecipitated contains ^{32}P indicating that the phosphorylation of GPBP occurs *in vivo* and therefore is likely to be a physiological process.

The rGPBP Is a Serine/Threonine Kinase That Phosphorylates the N-terminal Region of the Human GP Antigen—Although GPBP does not contain the 12 conserved structural regions required to define the classic catalytic domain for a protein kinase, the recent identification and characterization of novel non-conventional protein kinases (19–27) encouraged the investigation of its phosphorylating activity. Addition of [γ-^{32}P]ATP to rGPBP either from yeast or 293 cells (not shown) in the presence of Mn^{2+} and Mg^{2+} resulted in the incorporation of ^{32}P as Ser(P) and Thr(P) in the major and related products that were recognized by both anti-FLAG and specific antibodies (Fig. 5, *A* and *B*), indicating that the affinity purified material contains a Ser/Thr protein kinase. To characterize this activity further, GPpep1, GPpep1Ala^9 (a GPpep1 mutant with Ser^9 replaced by Ala), native and recombinant human antigens, and native bovine antigen were assayed (Fig. 5*C*). Affinity purified rGPBP phosphorylates all human-derived material to a different extent; however, in similar conditions no appreciable ^{32}P incorporation was observed in the bovine-derived substrate. The lower ^{32}P incorporation displayed by GPpep1Ala^9 when compared with GPpep1 and the lack of phosphorylation of the bovine antigen indicates that the kinase present in rGPBP discriminates between human and bovine antigens and that Ser^9 is a target for the kinase.

Although the purification system provides high quality material, the presence of contaminants with a protein kinase activity could not be ruled out. The existence of contaminants was also suggested by the presence of a FLAG-containing 40-kDa polypeptide displaying no reactivity with specific antibodies nor incorporation of ^{32}P in the phosphorylation assays (Figs. 4*A* and 5*A*). To identify precisely the polypeptide harboring the protein kinase activity, we performed *in vitro* kinase renaturation assays after SDS-PAGE and Western blot (Fig. 6). We successfully combined the use of specific antibodies (*lane 1*) and autoradiographic detection of *in situ* ^{32}P incorporation (*lane 2*), and we identified the 89-kDa rGPBP material as the primary

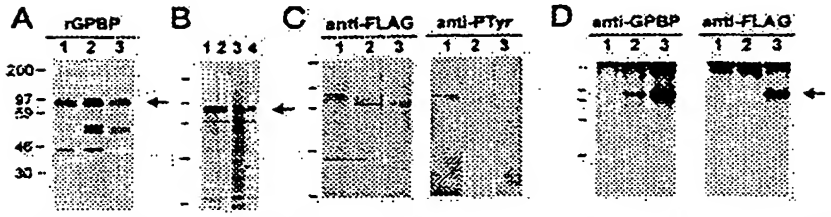

FIG. 4. **Characterization of rGPBP from yeast and 293 cells.** *A*, 1 μg (*lane 1*) or 100 ng (*lanes 2* and *3*) of yeast rGPBP were analyzed by reducing SDS-PAGE in a 10% gel. The separated proteins were stained with Coomassie Blue (*lane 1*) or transferred and blotted with anti-FLAG antibodies (*lane 2*) or monoclonal antibody 14, a monoclonal antibody against GPBP (*lane 3*). *B*, the cell extracts from GPBP-expressing yeast were analyzed as in *A* and blotted with anti-FLAG (*lane 1*), anti-Ser(P) (*lane 2*), anti-Thr(P) (*lane 3*), or anti-Tyr(P) (*lane 4*) monoclonal antibodies, respectively. *C*, 200 ng of either yeast rGPBP (*lane 1*), dephosphorylated yeast rGPBP (*lane 2*), or 293 cell-derived rGPBP (*lane 3*) were analyzed as in *B* with the indicated antibodies. *D*, similar amounts of $H_3{}^{32}PO_4$-labeled non-transfected (*lanes 1*), stable pc-n4'-transfected (*lanes 2*), or transient pc-FLAG-n4'-expressing (*lanes 3*) 293 cells were lysed, precipitated with the indicated antibodies, and analyzed by SDS-PAGE and autoradiography. The molecular weight markers are represented with *numbers* and *bars* as in Fig. 3. The *arrows* indicate the position of the rGPBP.

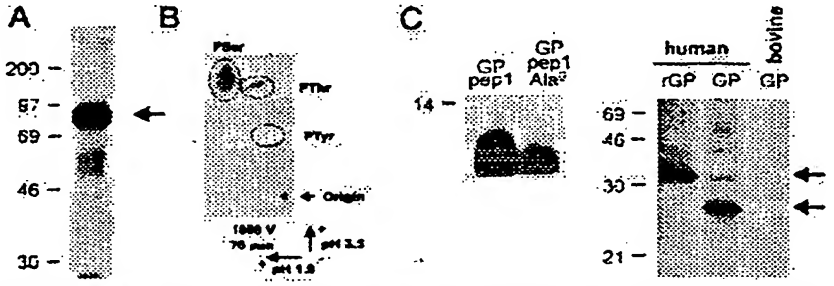

FIG. 5. **Recombinant GPBP contains a serine/threonine kinase that specifically phosphorylates the N-terminal region of the human GP antigen.** To assess phosphorylation, approximately 200 ng of yeast rGPBP were incubated with [γ-^{32}P]ATP in the absence (*A* and *B*) or presence of GP antigen-derived material (*C*). *A*, the mixture was subjected to reducing SDS-PAGE (10% gel) and autoradiographed. *B*, the mixture was subjected to ^{32}P-phosphoamino acid analysis by two-dimensional thin layer chromatography. The *dotted circles* indicate the position of ninhydrin-stained phosphoamino acids. *C*, the phosphorylation mixtures of the indicated GP-derived material were analyzed by SDS-PAGE (15% gel) and autoradiography (*GPpep1* and *GPpep1Ala*^{5}) or immunoprecipitated with monoclonal antibody 17, a monoclonal antibody that specifically recognizes GP antigen from human and bovine origin, and analyzed by SDS-PAGE (12.5%) and autoradiography (*rGP* and *GP*). The relative positions of rGPBP (*A*), rGP antigen, and the native human and bovine GP antigens (*C*) are indicated by *arrows*. The *numbers* and *bars* refer to molecular weight markers as in previous figures.

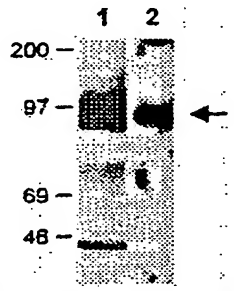

FIG. 6. **In-blot renaturation of the serine/threonine kinase present in rGPBP.** Five micrograms of rGPBP from yeast were in-blot renatured. The recombinant material was specifically identified by anti-FLAG antibodies (*lane 1*), and the *in situ* ^{32}P incorporation was detected by autoradiography (*lane 2*). The *numbers* and *bars* refer to molecular weight markers as in previous figures. The *arrow* indicates the position of the 89-kDa rGPBP polypeptide.

polypeptide harboring the Ser/Thr kinase activity. The lack of ^{32}P incorporation in the rGPBP-derived products as well as in the 40-kDa contaminant further supports the specificity of the renaturation assays and locates the kinase activity to the 89-kDa polypeptide. Recently, it has been shown that traces of protein kinases intimately associated to a polypeptide can be released from the blot membrane, bind to, and phosphorylate the polypeptide during the labeling step (28). To assess this possibility in our system, we performed the renaturation studies using a small piece of membrane containing the 89-kDa polypeptide either alone or together with membrane pieces representing the different regions of the blot lane. We observed similar ^{32}P incorporation at the 89-kDa polypeptide regardless of the co-incubated pieces (not shown), indicating that if there are co-purified protein kinases in our sample they are not phosphorylating the 89-kDa polypeptide in the renaturation assays unless they co-migrate. Co-migration does not, however, appear to be a concern since rGPBP deletion mutants displaying different mobilities also have kinase activities and could similarly be in-blot renatured (not shown).

Immunohistochemical Localization of the Novel Kinase—To investigate GPBP expression in human tissues, we performed immunohistochemical studies using specific polyclonal (Fig. 7) or monoclonal antibodies (not shown). Although GPBP is widely expressed in human tissues, it shows tissue and cell specificity. In the kidney, the major expression is found at the epithelial cells of the tubules and at the mesangial cells and podocytes of the glomerulus. At the lung alveolus, the antibodies display a linear pattern suggestive of a basement membrane localization along with staining of pneumocytes. Liver shows low expression in the parenchyma but high expression in biliary ducts. The expression at the central nervous system is observed in the white matter and not in the neurons of the brain. In testis, a high expression in the spermatogonium contrasts with the lack of expression in the Sertoli cells. The adrenal gland shows a higher level of expression at the cortical cells *versus* the medullar. In the pancreas, GPBP is preferentially expressed in Langerhans islets *versus* the exocrine moiety, and in prostate, GPBP is expressed in the epithelial cells but not in the stroma (Fig. 7). Other locations with high expression of GPBP are striated muscle, epithelial cells of intestinal tract, and Purkinje cells of the cerebellum (not shown). In general, in the tissues where GPBP is highly expressed the staining pattern is mainly diffuse cytosolic. However in certain

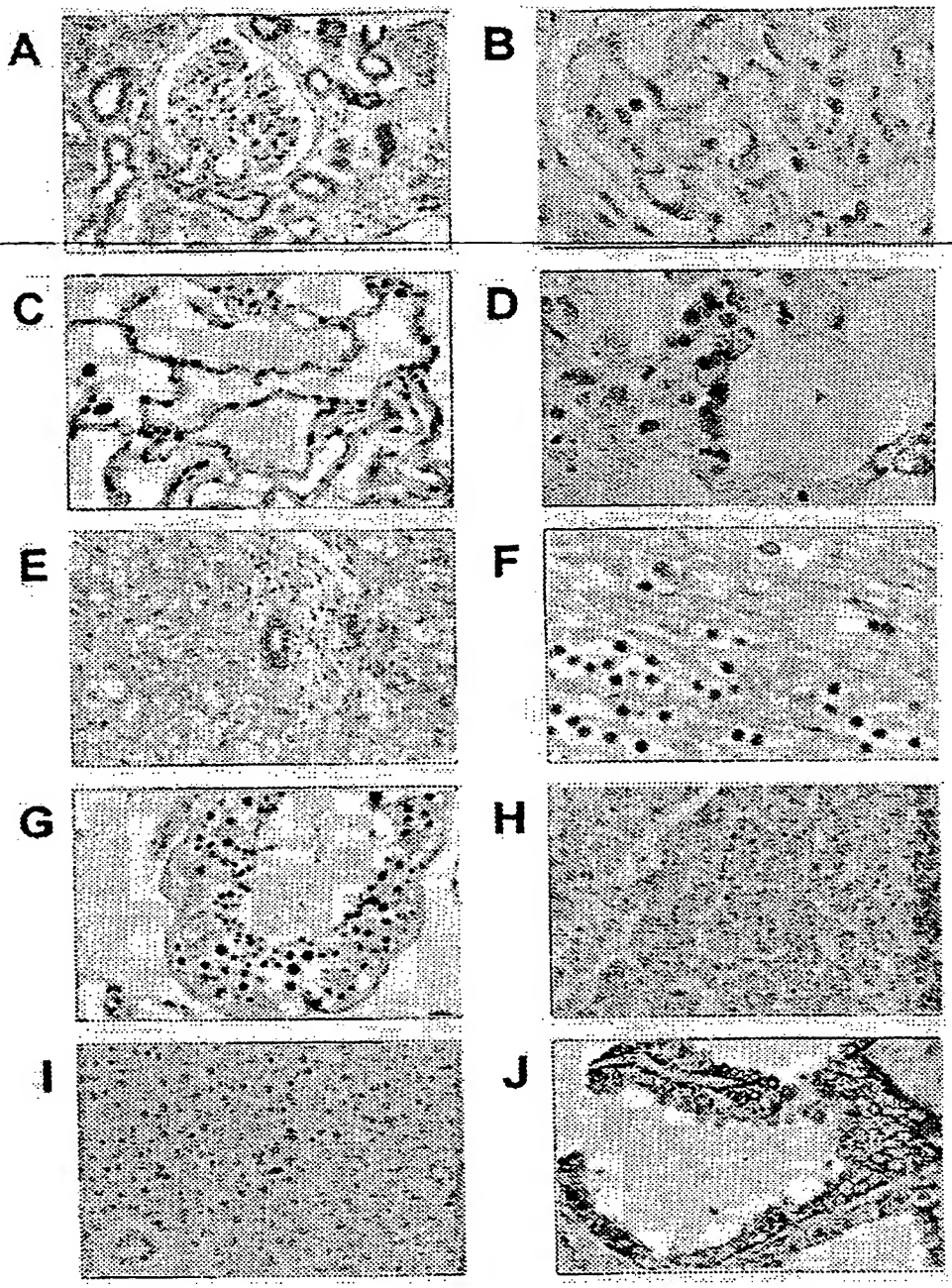

FIG. 7. **Immunological localization of GPBP in human tissues.** Rabbit serum against the N-terminal region of GPBP (1:50) was used to localize GPBP in human tissues. The tissues shown are as follows: kidney (*A*), glomerulus (*B*), lung (*C*), alveolus (*D*), liver (*E*), brain (*F*), testis (*G*), adrenal gland (*H*), pancreas (*I*), and prostate (*J*). Similar results were obtained using anti-GPBP affinity purified antibodies or a pool of culture medium from seven different GPBP-specific monoclonal antibodies (anti-GPBP monoclonal antibodies 3, 4, 5, 6, 8, 10, and 14). Rabbit preimmune serum did not stain any tissue structure in parallel control studies. Magnification was × 40 except in *B* and *D* where it was × 100.

locations there is, in addition, an important staining reinforcement at the nucleus (spermatogonium), at the plasma membrane (pneumocyte, hepatocyte, prostate epithelial cells, white matter), or at the extracellular matrix (alveolus) (Fig. 7).

DISCUSSION

Our data show that GPBP is a novel non-conventional serine/threonine kinase and present evidence that indicate that GPBP discriminates between human and bovine GP antigens and targets the exclusive human phosphorylatable region *in vitro*. Although the presence of additional protein kinases in the affinity-purified rGPBP cannot completely be ruled out, several lines of evidence indicate that the 89-kDa polypeptide is the only kinase therein. First, we found no differences in auto- or trans-phosphorylation among rGPBP samples purified either in the presence of 150 mM or 0.5, 1, or 2 M salt (not shown) suggesting that rGPBP does not carry kinases intimately bound. Second, the presence of a FLAG-containing yeast-derived kinase in our samples is not a concern since material purified using GPBP-specific antibodies show no differences in phosphorylation (not shown). Third, a deletion mutant of GPBP displays reduced auto- and trans-phosphorylation activities,[3] suggesting that is the only material in the rGPBP with the ability to carry out phosphate transfer.

Although GPBP is not homologous to other non-conventional kinases, they share some structural features including a N-terminal α-helix coiled-coil (26, 27), serine-rich motifs (24), high phosphoamino acids content (27), bipartite nuclear localization signal (27), and the absence of a typical nucleotide or

[3] A. Raya and J. Saus, unpublished observations.

ATP-binding motif (24, 27).

Immunohistochemistry studies show that GPBP is a cytosolic polypeptide also found in the nucleus, associated with the plasma membrane and likely at the extracellular matrix associated with basement membrane, indicating that it contains the structural requirements to reach all these destinations. The nuclear localization signal and the PH domain confer to it the potential to reach the nucleus and the cell membrane, respectively (17, 29, 30). Although GPBP does not contain the structural requirements to be exported, at the 5'-end untranslated region of its mRNA exists an upstream ORF of 130 residues with an in-frame stop codon at the beginning (Fig. 1). An mRNA editing process inserting a single base pair (U) would generate an operative in-frame start site and an ORF of 754 residues containing an export signal immediately downstream of the edited Met (not shown). Polyclonal antibodies against a synthetic peptide representing part of this hypothetical extra sequence display a linear vascular reactivity in human tissues suggestive of an extracellular basement membrane localization.[3] Alternatively, a splicing phenomenon could generate transcripts with additional unidentified exon(s) that would provide the structural requirements for exportation. The multiple cellular localization, the high content in Tyr(P), and the lack of tyrosine kinase activity *in vitro* suggest that GPBP in addition is the target of specific tyrosine kinase(s) and therefore likely involved in specific signaling cascade(s).

The idea that common pathogenic events exist at least for some autoimmune disorders is suggested by the significant number of patients displaying more than one autoimmune disease, and also by the strong and common linkage that some of these diseases show to specific major histocompatibility complex haplotypes (31, 32). The experimental observation that the autoantigen is the leading moiety in autoimmunity and that a limited number of self-components are autoantigenic (31) suggest that these components share biological features with important consequences in self/non-self recognition by the immune system. One possibility is that triggering events by altering different but specific self-components would result in abnormal antigen processing. In certain individuals expressing a particular major histocompatibility complex specificity, the abnormal peptides could be recognized by non-tolerized T cells and trigger an immune response (1).

We explored the GP antigen to identify biological features of relevance in autoimmune pathogenesis. Since the human antigen is a natural autoantigen but not the homologous counterparts from other superior mammals, and only $\alpha 3$ is involved in autoimmunity but not the remaining five α chains, comparative studies among NC1 domains were a useful initial approach. These studies revealed that specific serine phosphorylation as well as pre-mRNA alternative splicing are biological hallmarks of the human *versus* the other species GP antigens (4, 5). These two features are also associated with the biology of other autoantigens including acetylcholine receptor and myelin basic protein (4). The latter is suspected to be the major antigen in multiple sclerosis, another exclusively human autoimmune disease in which the immune system targets the white matter of the central nervous system. GP disease and multiple sclerosis are human disorders that display a strong association with the same HLA class II haplotype (HLA DRB1*1501) (32, 33). This along with the recent report of death by GP disease of a multiple sclerosis patient carrying this HLA specificity (34) support the existence of common pathogenic events in these human disorders.

Phosphorylation of specific serines has been shown to change intracellular proteolysis (35–40). Conceivably alterations in protein phosphorylation can affect processing and peptide presentation and thus mediate autoimmunity. GP antigen-derived peptide presentation by the HLA-DR15 depends more on processing than on preferences of relatively indiscriminate DR15 molecules (41), suggesting that if processing is influenced by abnormal phosphorylation, the resulting peptides would likely be presented by this HLA. Our more recent data indicate that in both the GP and myelin basic protein systems, the production of alternative splicing products serves to regulate the phosphorylation of specific and structurally homologous cAMP-dependent protein kinase sites,[2] suggesting that this or a closely related kinase is the *in vivo* phosphorylating enzyme. Alterations in the degree of antigen phosphorylation, caused either by an imbalance in alternative products or by the action of an intruding kinase that deregulates phosphorylation of the same motifs, could lead to an autoimmune response in predisposed individuals. Accordingly, we found that in kidney, GP patients express relatively more alternative products than control individuals (5) and that rGPBP phosphorylates the human GP antigen at a major cAMP-dependent protein kinase phosphorylation site in an apparently unregulated fashion since alternative products do not affect antigen phosphorylation.[3]

Although GPBP is ubiquitously expressed, in certain organs and tissues it shows a preference for cells and tissue structures that are a target of common autoimmune responses as follows: the Langerhans cells (type I diabetes); the white matter of the central nervous system (multiple sclerosis); the biliary ducts (primary biliary cirrhosis); the cortical cells of the adrenal gland (Addison disease); striated muscle cells (myasthenia gravis); spermatogonium (male infertility); Purkinje cells of the cerebellum (paraneoplastic cerebellar degeneration syndrome); and epithelial intestinal cells (pernicious anemia, autoimmune gastritis and enteritis). Although it is premature to draw definitive conclusions on the pathogenic involvement of GPBP, all the above observations point to this novel kinase as an attractive candidate to be considered when envisioning a model for human autoimmune disease.

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